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Original Article

Long term toxicity of chelerythrine on NF-κB expression in rat pulmonary tissues

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Abstract: Chelerythrine is one cytotoxic anti-tumor drug whose toxic effect is poorly understood. This study observed long-term toxicity of chelerythrine on tissue morphology and expression level of nuclear factor kappa B (NF-κB) in rat pulmonary tissues, to investigate related mechanism causing rat lung damage. Wistar rats were assigned into control, high (8.4 mg/kg), moderate (5.6 mg/kg) and low (3.7 mg/kg) dosage of chelerythrine groups (N=30 each). Three cycles of drug applications, each containing 6 days delivery and 8 days interval, were applied, followed by 4-week observation. General condition of rats was observed. Lung tissues were extracted, and were observed for tissue morphology by HE staining. Interleukin-6 (IL-6), IL-8 and tumor necrosis factor (TNF)-α levels were measured by ELISA. RT-qPCR and Western blot were used to quantify mRNA and protein levels of NF-κB and cell adhesion molecule ICAM-1. Accumulative mortality was highest in high dosage group, followed by moderate and low dosage group. Drug treatment decreased body weight and food intake of rats compared to control group. Body weight decrease was more significant with higher dosage. Drug treatment led to pulmonary congestion and bloody ascites. HE staining showed aggravated lung injury with higher dosage. Drug treatment group showed higher serum IL-6, IL-8 and TNF-α levels ($p<0.05$), and higher mRNA/protein expression of NF-κB and ICAM-1 in lung tissues in a dose-dependent manner ($p<0.05$). Chelerythrine has a long-term toxicity on lung tissues in a dose-dependent manner. Under high dosage (≥5.6 mg/kg), it can cause aggravate toxic lung damage, possibly via NF-κB activation and production of inflammatory cytokines.

Keywords: Chelerythrine, long term toxicity, NF-κB, pulmonary injury

Introduction

Chelerythrine is one benzo[c]phenanthridines alkaloid and has cytotoxic effects on multiple tumor cells including nasopharyngeal carcinoma cell KB, cervical cancer, gastric carcinoma and lung cancer cells [1, 2]. It can exert anti-tumor effects as to induce tumor cell apoptosis and to inhibit cell cycle via apoptosis factors, production of free oxygen radicals and inhibition of protein kinase C activity [3, 4]. *In vivo* study showed that chelerythrine could improve liver fibrosis in rats [5, 6]. Acute toxic assay showed significant toxicity response by chelerythrine, and can cause death via direct stimulation on abdominal organs, or acute respiratory distress. Long term toxic assay showed no suppression of bone marrow system, but can cause pathology injury of male rat testis, pulmonary congestion or bloody ascites, and can cause systemic response at ≥5.6 mg/kg [7, 8].

Inflammatory cascade reaction plays a crucial role in pulmonary tissue damage and pathology. Pulmonary alveolar epithelial cell, inflammatory cells and fibroblast can produce multiple cytokines for participating in inflammation and aggravating inflammatory damage. NF-κB, p38 mitogen activating protein kinase (MAPK) signal pathways are major transduction pathways. NF-κB has multiple regulatory functions, and can mediate gene transcription levels of various inflammatory mediators, cytokines and chemokines such as IL-8, IL-6 and ICAM, thus playing crucial role sin cell inflammation and apoptosis. Previous study showed that NF-κB was involved in lung tissue injury. Neutrophils

Table 1. Primer sequence

Primer	Size	Forward	Reverse
NF-κB mRNA	100 bp	5'-GAGAGCCCTTGCATCCTTTA-3'	5'-CTTCCCTTTGGTCTTTCTGT-3'
ICAM-1 mRNA	260 bp	5'-CGACTGGACGAGAGGGATTG-3'	5'-TTATGACTGCGGCTGCTACC-3'
β-actin	220 bp	5'-TGCTGTCCCTGTATGCCTCT-3'	5'-TTTGATGTCACGCACGATTT-3'

can adhere to the injury vascular endothelial cells, and translocate toward pulmonary alveolar cavity or mesenchyme, thus producing abundant inflammatory mediators such as leukotriene, inflammatory cytokines, and participating in neutrophils-induced pulmonary damage. ICAM-1 participates in inflammatory response and related pathology, and its expression level can be up-regulated by multiple inflammatory factors [9, 10]. Currently few studies have been performed regarding drug efficiency or toxicology of chelerythrine. To further investigate the toxicity and target organs of chelerythrine, this study observed the effect of long-term chelerythrine delivery on rat pulmonary tissue damage and expression levels of NF-κB and ICAM-1, thus investigating the role of long-term chelerythrine toxicity in rat lung tissues and related mechanisms.

Materials and methods

Animals and grouping

Healthy Wistar rats (both males and females, body weight 120-140 g) were provided by Laboratory Animal Center, Chinese Medical Academy (Certificate No., SYXK-2013-0025) and kept in a SPF grade animal facility with standard food and water. Rats were randomly assigned into control, high (8.4 mg/kg), moderate (5.6 mg/kg) and low (3.7 mg/kg) dosage of chelerythrine groups (N=30 each). Three cycles of intra-peritoneal drug applications, each containing 6 days of drug delivery and 8 days of interval, were applied, followed by 4-week observation. Drug volume was 1 ml/100 g. Control group received equal volume of saline, whilst group B, C and D received 8.4 mg/kg, 5.6 mg/kg and 3.7 mg/kg of chelerythrine.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the third Xiangya Hospital of Central South University.

Drugs and reagents

Chelerythrine (condensed solution, 10 mg/ml, 250 ml) was provided by Ouhua Pharmaceutical and diluted in saline before use. Urethane was purchased from Sigma (US). Interleukin-6 (IL-6) and IL-8 test kits were provided by Jiancheng Bio (China). Primers for NF-κB and ICAM-1 were provided by Invitrogen (US). Antibody for NF-κB and ICAM-1 proteins, secondary antibody, reverse transcription kit and Bioneer amplification kit were purchased from Zhongshan Jinqiao Biotech (China).

Drug dosage

Dosage of chelerythrine was determined according to the previous literature and acute toxicology study, which showed LD_{50} value at 24.5 mg/kg in mouse by intra-peritoneal injection, whilst effective dosage was 2 mg/kg in mice. We thus assigned 8.4, 5.6 and 3.7 mg/kg as high, moderate and low dosage, which equals to 11-fold of that on humans.

Long term toxicity assay

Following generally accepted guideline of cytotoxicity study in anti-tumor drugs, rats received daily intra-peritoneal injection of drugs for 3 cycles, each consisted for 6 days drug delivery followed by 8 days interval. After suspension of drugs, rats were continuously monitored for 4 weeks to observe general condition for recording mortality rate.

ELISA

Rat serum levels of IL-6, IL-8 and TNF-α were measured following the manual instruction of the corresponding test kit. Absorbent (A) values at 450 mm were measured on a microplate reader.

HE staining for lung tissue morphology

Rats were sacrificed and dissected to observe the general morphology of lung tissues, which were immersed in paraformaldehyde and embedded in paraffin to prepare 5 μm slices. After hematoxylin-eosin staining, tissues were observed under a light field microscope.

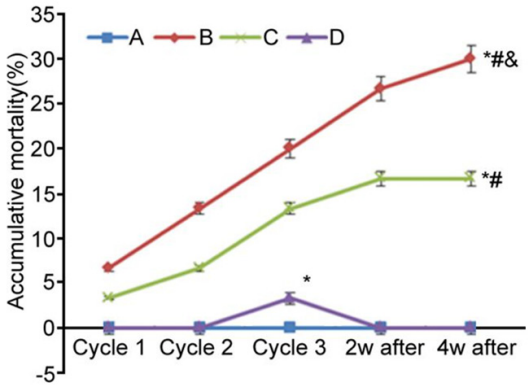

Figure 1. Accumulative mortality of rats. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

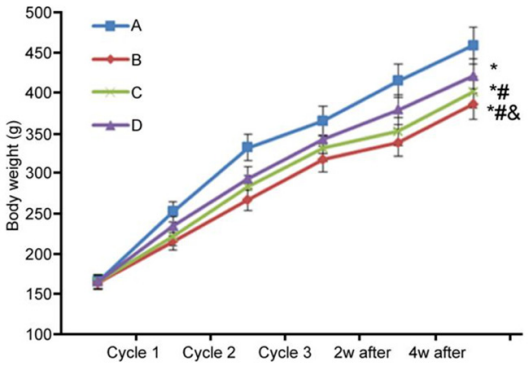

Figure 2. Effects of chelerythrine on rat body weight. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

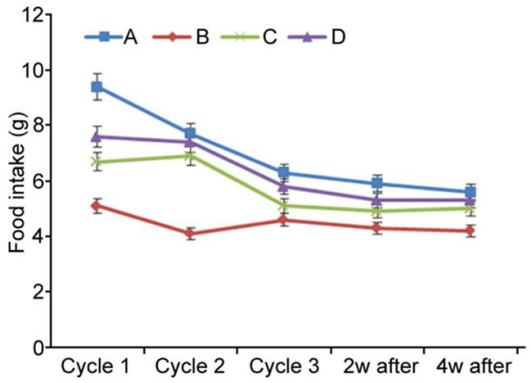

Figure 3. Effects of chelerythrine on rat food intake. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group.

qRT-PCR for NF-κB and ICAM-1 mRNA expression in lung tissues

Lung tissues were collected for preparing homogenate. TransZol Up approach was employed to quantify total RNA. Using reverse transcription cDNA as the template, PCR amplification was performed under the following conditions: 95°C pre-denature for 10 min, followed by 40 cycles each containing 95°C denature 10 s, 55°C annealing 30 s and 72°C elongation 45 s. Using beta-actin as the internal reference, semi-quantitative analysis was performed using Rotor-Gene Q Series Software. Each sample was tested in triplicates, using primers as shown in **Table 1**.

Western blot for NF-κB and ICAM-1 protein expression

100 mg lung tissues were collected to extract protein in RIPA lysis buffer. Protein concentration was measured by Bradford method. Proteins were separated in SDS-PAGE, and were transferred to PVDF membrane, which was blocked in defatted milk powder. Primary monoclonal antibody against NF-κB or ICAM-1 (1:100 dilution) was added for overnight incubation. Following TBST rinsing, secondary antibody (1:200 dilution) was added for 1 h incubation. Color was developed and exposed in dark. Quantity One imaging analysis software was used to analyze colored bands. Relative expression level of was presented as protein band density value against that of β-actin.

Statistical methods

SPSS 19.0 software was used for analysis. Those data fitted normal distribution were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and LSD test were performed. A statistical significance was defined when $p<0.05$.

Results

General condition of rats

After drug treatment, high dosage group immediately showed body twisting and positive signs of peritoneal irritation lasting for up to 40 min. After one cycle of drug treatment, ascites were developed. During drug intervention period, all

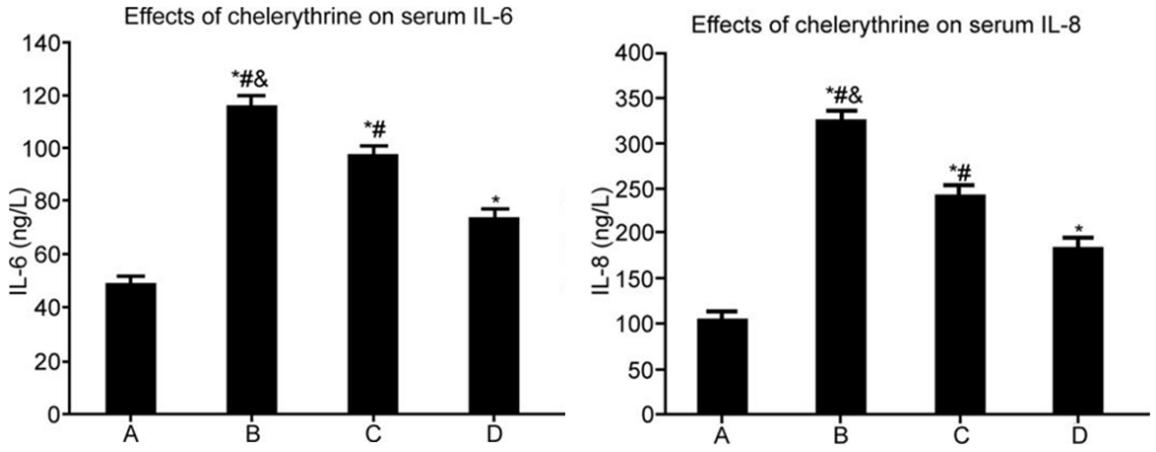

Figure 4. Effects of chelerythrine on rat serum IL-6 and IL-8. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

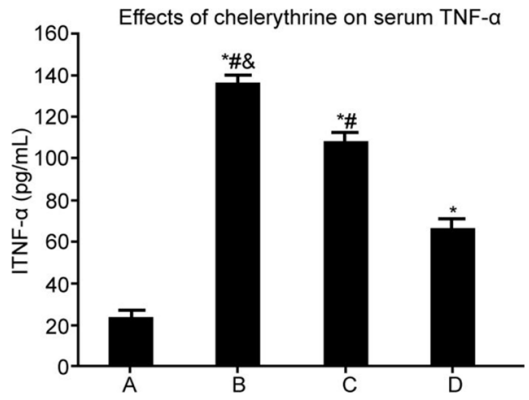

Figure 5. Effects of chelerythrine on rat serum TNF-α. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

treatment groups showed significantly lower body weight or food intake, with more potent difference with higher dosage (p<0.05). Drug treatment also caused pulmonary congestion and blood ascites. Accumulative mortality was highest in group B (high dosage group), followed by group C (moderate dosage) and group D (low dosage), as shown in **Figures 1**-**3**.

Effects of chelerythrine on rat serum IL-6, IL-8 and TNF-α level

Compared to group A, all treatment groups (group B, C and D) showed elevated serum levels of IL-6, IL-8 and TNF-α (p<0.05), whilst IL-6, IL-8 and TNF-α levels were elevated with higher dosage, indicating dose-dependent manner (**Figures 4** and **5**).

Effects of chelerythrine on rat pulmonary morphology

HE staining showed no significant abnormality in rat lung tissues in control group, whilst drug treatment group showed aggravated pulmonary tissue damage with higher dosage, and no complete recovery after drug retrieval. Inflammatory infiltration was observed during drug delivery period in high, moderate or low dosage groups. High dosage group showed thickening of pulmonary alveolar wall, whilst moderate or low dosage showed alleviated damage (**Figure 6**).

Effects of chelerythrine on rat pulmonary NF-κB and ICAM-1 expression

Compared to group A, all drug treatment groups (group B, C and D) showed enhanced mRNA and protein expression levels of NF-κB and ICAM-1 in pulmonary tissues (p<0.05) with a dose-dependent manner, as shown in **Figures 7**-**9**.

Discussion

This study observed the long-term toxicity of chelerythrine on rat and targeted organs. Results showed that continuous delivery of chelerythrine exerted certain damage on lung tissues, as drug treatment groups showed pul-

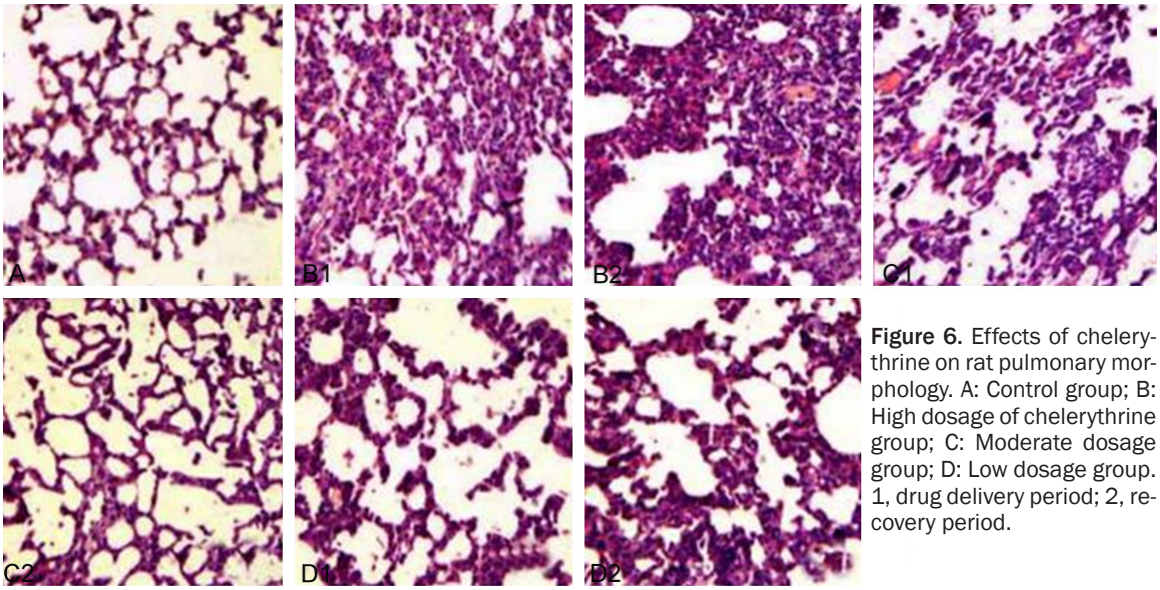

Figure 6. Effects of chelerythrine on rat pulmonary morphology. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. 1, drug delivery period; 2, recovery period.

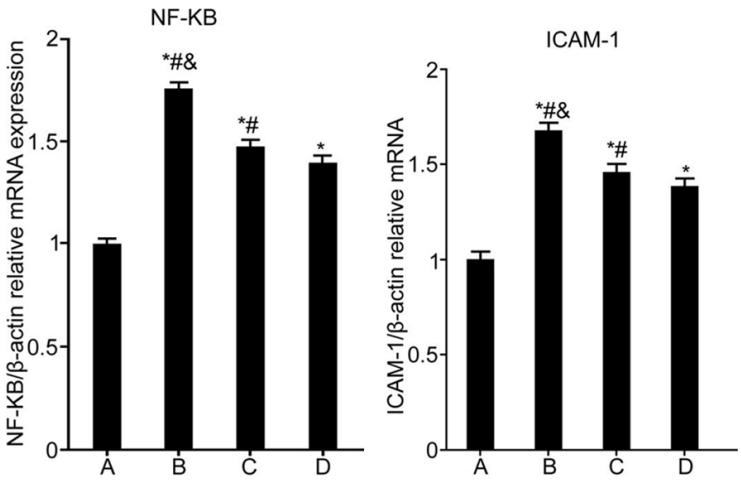

Figure 7. Effects of chelerythrine on rat pulmonary NF-κB and ICAM-1 mRNA expression. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

monary congestion and blood ascites. The condition of pulmonary damage was aggravated with higher dosage, and cannot completely recovered after drug retrieval. These results suggested long-term toxicity of chelerythrine on pulmonary tissues in a dose-dependent manner. With ≥5.6 mg/kg dosage, it can cause pulmonary damage related with drug toxicity, which was aggravated with higher dosage.

Inflammatory damage plays a critical role in both acute and chronic lung injury. IL-6 mainly derives from mononuclear macrophage, endothelial cells, vascular smooth muscle cells, T/G lymphocytes, and can facilitate ICAM-1 gene expression in lung tissues. The up-regulation of ICAM-1 mRNA can bind with surface integrin LFA-1 on neutrophil to initiate signal transduction facilitating adhesion between neutrophils and endothelial cells for induction of neutrophil infiltration [11-13]. IL-8 is one inflammatory cell chemokine for T lymphocyte and neutrophil, and mainly derives from epithelial cells, macrophage and neutrophil. Il-6 can stimulate the production of IL-8, which can bind with cell surface G-protein coupled receptor (GPCR) for cellular signal transduction, inducing leukocyte adhesion, activation and facilitating binding of leukocyte onto extracellular matrix (ECM) proteins to release protein hydrolysis enzyme and peroxide, thus causing pulmonary tissue damage [14, 15]. This study showed elevated serum levels of IL-6 and IL-8 in chelerythrine treated rats in a dose-dependent manner, indicating that chelerythrine had a long-term toxicity for up-regulating rat serum IL-6 and IL-8 levels to facilitate inflammatory injury.

Multiple cellular signal pathways can regulate production and release of inflammatory mediators including IL-6 and IL-8. NF-κB is one crucial

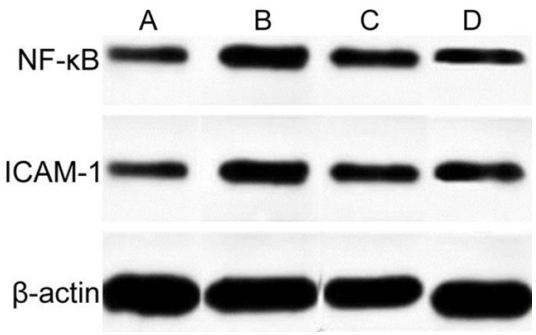

Figure 8. Effects of chelerythrine on rat pulmonary NF-κB and ICAM-1 protein expression. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

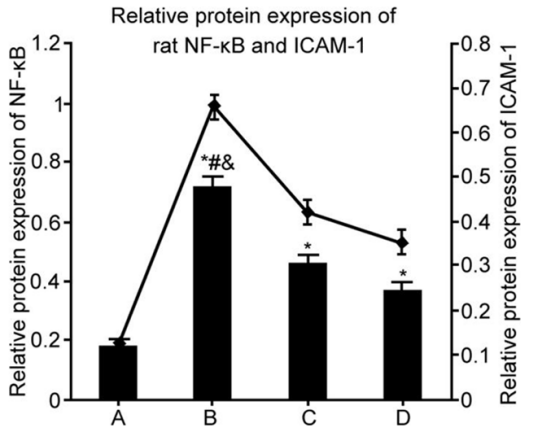

Figure 9. Effects of chelerythrine on rat pulmonary NF-κB and ICAM-1 expression. A: Control group; B: High dosage of chelerythrine group; C: Moderate dosage group; D: Low dosage group. *, P<0.05 compared to group A; #, P<0.05 compared to group D; &, P<0.05 compared to group C.

pathway, and is involved in acute pulmonary injury [16, 17]. Both *in vivo* and *in vitro* assays showed the participation of NF-κB in pathological process of pulmonary tissue injury [18, 19]. Usually cytoplasm has no activity of NF-κB, which exists in the form of binding with inhibitory protein IκB. After NF-κB activation, IκBα expression was facilitated, for a further feedback regulation on NF-κB expression [20, 21]. IL-1 and LPS signals cause IκB degradation, for the release and further activation of NF-κB, which enters into the nucleus to specifically bind with κB binding sequence, to facilitate transcription of inflammatory mediator and cytokine, and production of pro-inflammatory factor and inflammatory mediator, thus leading to inflammatory response. The critical step in early phase of acute inflammatory injury is probably correlated with degradation of IκB after phosphorylation, and translocation of NF-κB from the complex [18, 22]. NF-κB binding sequence is abundantly expressed in upstream promoter and enhancer of adhesion molecule ICAM-1 and inflammatory mediator IL-8 genes, and its up-regulation can activate expression level of related genes [19, 21]. ICAM-1 is the ligand of integrin LFA-1, and can facilitate adhesion function between endothelial cells and leukocytes. This study showed that continuous delivery of chelerythrine enhanced mRNA expression of NF-κB and ICAM-1 in rat pulmonary tissues in a dose-dependent manner, indicating that chelerythrine can enhance NF-κB mRNA expression and facilitate adhesion function between leukocyte and endothelial cells or inflammatory response via potentiating expression of inflammatory factors. Results of this study also showed up-regulation of NF-κB and ICAM-1 protein expression in chelerythrine treated pulmonary tissues in a dose dependent manner for facilitating lung tissue injury. Long-term delivery of chelerythrine may exert certain toxicity on lung tissues, probably related with NF-κB signal pathway, as it can induce inflammatory damage via facilitating expression of inflammatory factors. This study, however, only considered the effect of long-term toxicity of chelerythrine on rat lung tissue morphology and NF-κB signal pathway, but leaving detailed mechanisms and potential effects on other signal pathways unattended.

Long-term toxicity of chelerythrine can produce dosage-dependent effects on pulmonary tissues. With ≥5.6 mg/kg dosage, it can cause pulmonary damage on rats, with aggravated injury in higher dosage, probably related with NF-κB activation and production of inflammatory cytokine production.

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Disclosure of conflict of interest

None.

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